Research Article

Isolation and characterization of bioactive compounds for *Bacillus cereus* and *Bacillus subtilis* from *Oreochromis mossambicus* and *Labeo rohita*

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ABSTRACT

The bioactive compounds production by fish probiotic bacteria *Bacillus cereus* SVSK2 (*B. cereus)* and *Bacillus subtilis* SVSK5 (*B. subtilis*) was analyzed by FT-IR, HPLC, HPTLC and GC-MS. In addition, the *in vitro* antibacterial activity was identified against clinical pathogens and anticancer activity of the probiotic bioactive compounds was analyzed by MTT assay using MCF-7, Hela and Vero cells. The identified bioactive compounds show highest antibacterial activity against *Klebsiella, E.coli, Serratia Proteus, V. cholerae, V. parahaemolyticus* and *V. harveyi* and anticancer activity against MCF-7, Hela and Vero cells by *in vitro*. The results revealed that the bioactive compounds shows good anticancer activity in MCF-7 compared with Hela cells. Vaigai results concluded that the *B. cereus* SVSK2 and *B. subtilis* SVSK5 from fish gut is a hopeful source of natural bioactive compounds which may gain great attention as promising sources of new drug development in the pharmacological industries.

Keywords: *Oreochromis mossambicus*; *Labeo rohita*; Probiota; *Bacillus* sp; bioactive compounds; Cytotoxicity.

INTRODUCTION

Probiotics are living microorganisms which when administered in adequate amounts provide health benefits to the host[1]. The fish gastro intestinal tract (GI) is populated with complex microbial community and it plays a vital role in promoting the health of the host through the production of secondary metabolites. Probiotic bacteria are known to produce bioactive substances thus protecting themselves against predators[2]. These microbial bioactive substances exhibit antibacterial, antiviral, anti-tumor and cardio protective properties[3, 4]. Probiotic bacteria may produce types of secondary metabolites. The bacterial bioactive compounds are used to inhibit the growth of the human and fish pathogens. However the fish microbial secondary metabolites production altered due to the anthropogenic stresses such as oil spills in water and pollution[5, 6].

Bacterial secondary metabolites or bioactive compounds having structural diversity obtained from natural sources enhance the biological activity of the host. The products of the bioactive compounds are used widely in cancer chemotherapy[7]. The Bioactive compounds having anticancer activity are extracted from microorganisms, terrestrial plants and marine life forms[8-10]. In our present study we revealed the therapeutic evaluation of the bioactive compounds produced by *B. cereus* SVSK2 and *B. subtilis* SVSK5 isolated from gastro-intestinal tract of *Oreochromis mossambicus* and *Labeo rohita*.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Condition

Previously identified *B. cereus* SVSK2 (accession number KU167636) (from *Oreochromis mossambicus*) and *B. subtilis* SVSK5 (accession number KU167639) (from *Labeo rohita*) *Vaigai* was used for this study. The selected colonies were inoculated in nutrient agar plates and incubated at ambient temperature for 24 h[11]. The glycerol stocks of individual isolates were maintained under deep freezer (-20°C) for further use [12].

Preparation of crude cell free extracts

The crude cell free extract of each of the selected isolates were obtained by first growing them in separate sets of 50 ml of nutrient broth at 37 ± 2°C for 16 to 18 hours culture, followed by centrifugation at 10,000 rpm for 15 min at 4°C after centrifugation 2:1 ratio ethyl acetate was added to separate bioactive compounds and subsequent filtration of each supernatant through 0.2 µm membrane under aseptic conditions.

Fourier transform infra-red spectra

IR spectrum was recorded in spectrophotometer (Shimadzu), the active principle was mixed with KBr and pellet technique was adopted to record the spectra [13].

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

The bioactive compounds were extracted from the harvested bacterial culture by centrifugation at 6000 rpm for 15 min in 4°C condition. The centrifuged cell supernatant was frozen overnight at -20 °C. Further the purification was done by chromatographic separation method and it was carried out with GC-MS-QP 2010 with and the specifications of the column was Db 30.0 column the diameter 0.25 µm × 0.25 µm thickness. The oven temperature was programmed, by the following conditions, from 70°C to 200°C with an increase of 10°C / min (isothermal for 5 minutes), in continuation the temperature 5°C / min to 280°C, ending with a 35 minute isothermal at 280°C. Mass spectra were measured at 70 eV; scan interval of 0.5 seconds, scan ranges from 40–1000 m / z. Helium was used as a carrier gas at 99.99%, the pressure flow 1.0 ml / min retention time, mass spectrum and the concentration of extract.

Therapeutic application

Antibacterial activity

The antibacterial activity of the Bioactive compounds were measured using agar disc diffusion assay against human pathogens such as *Klebsiella* (MTCC7407), *E.coli* (MTCC1303), *Serratia* (MTCC7103), *Proteus* (MTCC9493), *Vibrio cholerae* and the Fish pathogens such as *Vibrio harveyi, Vibrio parahaemolyticus* were used for the antibacterial activity assays.[14]

Cell lines

For the cytotoxicity studies the following cell lines was obtained from National Centre for Cell Science (NCCS), Pune. Human cervical cancer cell line (HeLa) , Breast cancer cell line (MCF-7) and Vero cell line (Normal) are cultured in Eagles Minimum Essential Medium containing 10% Fetal bovine serum (FBS) the cell lines were maintained by the following culture conditions ,incubated at 37°C, supplemented with 5% CO_2 and 95% air with the relative humidity of 100%.

Cytotoxicity assay

The morphological changes of the above mentioned cell lines was measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay method. The MTT was added to the medium at the final concentration was 0.5 mg/ml and further incubated for 4h in a humidified atmosphere at 37 °C with 5% CO_2. The growth media was removed from the wells leaving formazone crystals at the bottom, and the crystals were further dissolved in 200 µl with dimethyl sulfoxide the resulted absorbance was recorded at 570 nm immediately. Optical density (OD) values of each well were normalized against the control wells in without treatment.

RESULTS AND DISCUSSION

Probiotics are generally beneficial microorganisms that improve the health of the host because of their secondary metabolites or bioactive compounds production. In our previous study we had isolated the probiotic bacteria *B. cereus* SVSK2 and *B. subtilis* SVSK5 from the gut of fish samples collected from the river *Vaigai* [11]. Specifically, the bacteria *Bacillus* sp. produces natural bioactive secondary metabolites which have high antimicrobial and antifungal effects [15]. With this regard, in this study, the secondary metabolites production was evaluated in the isolates namely *B. cereus* SVSK2 and *B. subtilis* SVSK5.

FT IR Analysis

The IR results showed the characteristic features of aliphatic compounds with one or more C=C groups. The major peaks were at 1020.38 (C-H) stretch, 1247.99 (C=N) stretch, 1637.62 (C=C), 2075.47 (C=O) stretch and 3460.41 cm^{-1} that can be attributed to O-H stretch (Table 1; Fig 1). These results are concordant with previously identified secondary metabolites functional groups results[16, 17].

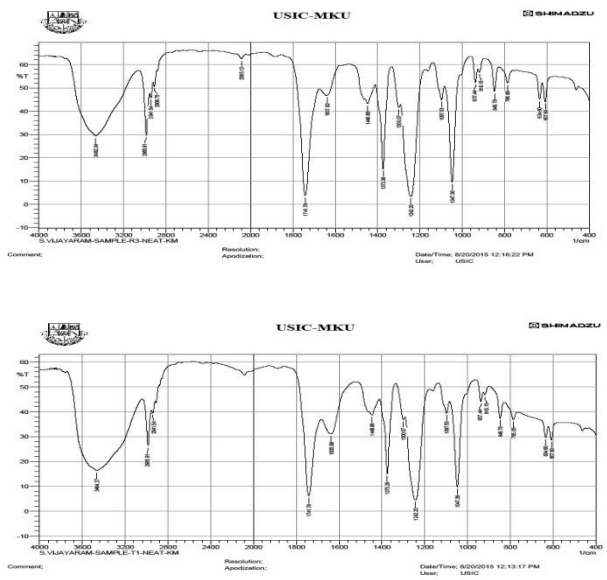


Figure: 1 FT IR analysis of bacterial sample

The HPLC results for SVSK2 and SVSK5 strains bioactive compounds. Totally ten peaks were observed in each bacterial cell free extracts and table 2 represents the retention time and percentage of each peaks. In addition HPTLC was used for the assessment of the effectiveness of the fractionation step. Table 3 shows Rf values of all fractions of SVSK2 and SVSK5 samples. It is obvious that the active compounds extracted are phenolic compounds. However, coumarin, caffeic acid and some unknown compounds were also found in the samples of SVSK2 and SVSK5.

GC-MS Analysis

In order to identify the structural elucidation of bioactive compounds, the GC-MS analysis was carried out. The GC analysis revealed the major bioactive compounds of

SVSK2 and SVSK5 (Table 4). It is our hypothesis that the increased levels of hydrocarbon contamination in *Vaigai* river due to oil spills, Industry wastes and sewage that would have increased the secretion of bioactive compounds and its derivatives as a defense mechanism to combat cellular damage [18,19].

B. cereus SVSK2			B. subtilis SVSK2		
Peak value	**Assignment and intensity**	**Functional groups**	**Peak value**	**Assignment and intensity**	**Functional groups**
607.6	C-Br	Alkyl halides	607.6	C-Br	Alkyl halides
634.6	C-Br	Alkyl halides	634.6	C-Br	Alkyl halides
786.98	C-C-H;CH	Alkyl halides	785.98	C-C-H;CH	Alkyl halides
846.78	C-C-H;CH	Alkenes	846.78	C-C-H;CH	Alkenes
918.15	N-H	Carboxylic acid	918.15	N-H	Carboxylic acid
937.44	N-H	Carboxylic acid	937.44	N-H	Carboxylic acid
1047.38	C-N stretch	Aliphatic amines	1047.38	C-N stretch	Aliphatic amines
1097.53	C-N stretch	Aliphatic amines	1097.53	C-N stretch	Aliphatic amines
1242.2	C-N stretch	Aliphatic amines	1242.2	C-N stretch	Aliphatic amines
1300.07	C-H wag	Alkyl halides	1300.07	C-H wag	Alkyl halides
1373.36	C-H rock	Alcohol, carboxylic acid ,ethers	1375.29	C-H rock	Alcohol, carboxylic acid ,ethers
1446.66	C-C stretch	Alkenes	1446.66	C-C stretch	Alkenes
1637.62	N-H bend	Amines	1635.69	N-H bend	Amines
1741.78	C=O stretch	Carboxylic acid	1741.78	C=O stretch	Carboxylic acid
3462.34	O-H stretch	Alcohol, phenols	3464.27	O-H stretch	Alcohol, phenols
B. cereus SVSK2			B. subtilis SVSK2		
Peak value	Assignment and intensity	Functional groups	Peak value	Assignment and intensity	Functional groups
607.6	C-Br	Alkyl halides	607.6	C-Br	Alkyl halides
634.6	C-Br	Alkyl halides	634.6	C-Br	Alkyl halides
786.98	C-C-H;CH	Alkyl halides	785.98	C-C-H;CH	Alkyl halides
846.78	C-C-H;CH	Alkenes	846.78	C-C-H;CH	Alkenes
918.15	N-H	Carboxylic acid	918.15	N-H	Carboxylic acid
937.44	N-H	Carboxylic acid	937.44	N-H	Carboxylic acid
1047.38	C-N stretch	Aliphatic amines	1047.38	C-N stretch	Aliphatic amines
1097.53	C-N stretch	Aliphatic amines	1097.53	C-N stretch	Aliphatic amines
1242.2	C-N stretch	Aliphatic amines	1242.2	C-N stretch	Aliphatic amines
1300.07	C-H wag	Alkyl halides	1300.07	C-H wag	Alkyl halides
1373.36	C-H rock	Alcohol, carboxylic acid ,ethers	1375.29	C-H rock	Alcohol, carboxylic acid ,ethers
1446.66	C-C stretch	Alkenes	1446.66	C-C stretch	Alkenes
1637.62	N-H bend	Amines	1635.69	N-H bend	Amines
1741.78	C=O stretch	Carboxylic acid	1741.78	C=O stretch	Carboxylic acid
3462.34	O-H stretch	Alcohol, phenols	3464.27	O-H stretch	Alcohol, phenols

Table 1: FT-IR absorption and functional groups for SVSK2 and SVSK5

Table 2: HPLC analysis of bioactive compounds isolated from SVSK2 and SVSK5 strains GI tract

B. cereus SVSK2							B. subtilis SVSK5						
S. No	Retention time (min)	Area (mV.s)	Height (Mv)	Area (%)	Height (%)	W05 (min)	S. No	Retention time (min))	Area (mV.s)	Height (Mv)	Area (%)	Height (%)	W05 (min)
1	2.073	93.331	14.734	11.0	16.4	0.10	1	2.177	121.464	16.913	12.0	15.2	0.12
2	2.203	351.624	45.167	41.6	50.3	0.09	2	2.303	425.801	60.591	42.0	54.5	0.09
3	2.623	119.830	7.599	14.2	8.5	0.27	3	2.677	127.351	8.095	12.6	7.3	0.26
4	2.957	40.724	3.035	4.8	3.4	0.24	4	3.023	52.243	3.995	5.2	3.6	0.23
5	3.330	22.851	1.796	2.7	2.0	0.26	5	3.377	24.633	2.035	2.4	1.8	0.24
6	3.567	100.444	10.021	11.9	11.2	0.15	6	3.610	100.726	10.305	9.9	9.3	0.15
7	3.850	32.587	2.821	3.9	3.1	0.20	7	3.880	36.414	3.260	3.6	2.9	0.20
8	4.180	24.711	1.546	2.9	1.7	0.21	8	4.187	31.170	1.974	3.1	1.8	0.21
9	5.603	31.512	1.960	3.7	2.2	0.26	9	5.490	31.862	2.056	3.1	1.8	0.25
10	8.183	27.427	1.169	3.2	1.3	0.40	10	7.903	62.023	1.947	6.1	1.8	0.42

Table 3: HPTLC results of bioactive compounds from isolates SVSK2 and SVSK5

B. cereus SVSK2				
Peak	Rf	Height	Area	Assigned substance
1	0.76	32.2	1019.6	Caffeic acid
2	0.8	23.2	536.6	Coumarin
3	0.95	17.9	659	Unknown
STD	0.76	588.7	11610.5	Phenolic standard (Quercetin)
B. subtilis SVSK5				
1	0.78	93.7	1715.5	Phenolic compound
2	0.85	26.9	559.8	Coumarin
3	0.95	33.1	1377.3	Unknown
STD	0.76	588.7	11610.5	Phenolic standard (Quercetin)

Nevertheless, it is evident that the bioactive compounds of SVSK2 and SVSK5 may include some potent chemotherapeutic substances, notably antibiotics mediated by free radical scavenging effect [20], antioxidant effect and some potent anticancer principles that include bioactive compounds. So, to identify their therapeutic potential, antimicrobial and anticancer effects *in vitro* evaluation was done.

Antibacterial and Anticancer activity

With the intention of identifying the therapeutic applications of SVSK2 and SVSK5 bacterial isolates cell free extracts, an antibacterial activity was identified against clinical pathogens and *in vitro* anticancer effect of bioactive compounds was examined in human cervical cancer (HeLa) and human breast cancer cell line (MCF-7). The antibacterial effect of the crude cell free extracts of SVSK2 and SVSK5 against selected human and fish pathogen shows efficient inhibitory activity against human pathogens such as *Escherichia coli* (MTCC1303), *Klebsiella* (MTCC3384), *Bacillus* (MTCC6428), *Proteus mirabilis* (MTCC9493), *Serratia marcescens* (MTCC7103), *Staphylococcus aureus* (MTCC7405). However, the SVSK2 crude cell free extract shows significant inhibitory activity against *Serratia*, *Proteus mirabilis*, *Klebsiella* and *Escherichia coli* when compared to SVSK5 activity. The SVSK2 crude cell free extract shows significant inhibitory activity against *Vibrio* species too (Fig. 2). As per the previous studies, the probiotics have inhibitory effects on the growth of a wide range of intestinal pathogens in human. The probiotics like *Lactobacillus*, *Bacillus spp.* and *Streptococcus* spp. in addition have defensive effect against the development of colon tumors [21]. These results suggested that the isolated bacterial strains come under probiotics due to the inhibitory activity of its byproducts against pathogens. Table 4 also shows the therapeutic applications of the isolated compounds.

Table 4: GCMS results for SVSK2 and SVSK5

		SVSK2				SVSK5		
Peak No	**Compounds**	**Molecular Formula**	**Therapeutic applications**	**References**	**Compounds**	**Molecular Formula**	**Therapeutic applications**	**References**
1	2-Butanone, 4-Hydroxy-3-Methyl-	$C_5H_{10}O_2$	anti-microbial, anticancer	Wei et al 2008 Wang et al 2014	Cycloserine	$C_3H_6N_2O_2$	anti-bacterial	*Prosser et al 2013*
2	Isobutyl Acetate	$C_6H_{12}O_2$	anti-fungal, anti-microbial	Len et al 2016	Neopentyl Glycol	$C_5H_{12}O_2$	anti-microbial	
3	Methoxyacetic Acid, 3-Tetradecyl	$C_{17}H_{34}O_3$	not yet identified	Vaithiyanathan et al 2015	Ethanol, 2-(Dodecyloxy	$C_{14}H_{30}O_2$	anti-tumor anti-bacterial anti-cancer	Flora et al 2013
4	Phenol, 2,4-Bis(1,1-Dimethylethyl)	$C_{14}H_{22}O$	anti-fungal anti-microbial antioxidant	Rangel-Sánchez 2014	Heptadecane, 2,6,10,15-Tetramethyl	$C_{17}H_{36}$	antioxidant anti-tuberculosis anti-bacterial	Varsha Jadhav et al 1014
5	Hentriacontane	$C_{31}H_{64}$	antioxidant anti-inflammatory anti-fungal	Jeffery et al 1983	Phenol, 2,5-Bis(1,1-6dimethylethyl	$C_{14}H_{22}O$	anti-inflammatory free radical scvanging	Rangel-Sánchez 2014
6	Nonadecane, 9-Methyl	$C_{20}H_{42}$	anti-microbial anti-fungal anti-diabetic antioxidant	Radhamani T and S. John Britto 2013	Hexacosane	$C_{26}H_{54}$	anti-inflammatory antioxidant anti-microbial	Amalraj , Ignacimuthu 1998
7	Oxalic Acid, 6-Ethyloct-3-Yl Hep	$C_{11}H_{20}O_4$	anti-microbial anti-inflammatory	Premlata Singariya et al 2015	Eicosane, 10-Methyl	$C_{21}H_{44}$	anti-cancer anti-tumor antioxidant	Hsouna et al 2011
8	Nonacosane	$C_{29}H_{60}$	anti-inflammatory	Cuauhtemoc Pérez González et al 2013	Pentacosane	$C_{25}H_{52}$	anti-cancer anti-tumor antioxidant	De Martino et al 2009
9	Sulfurous Acid, Butyl Dodecyl Ester	$C_{16}H_{34}O_3S$	diabetics anthelmintic antibacterial antifungal	Vadivel and Gopalakrishnan, 2011	Phthalic Acid, Bis(7-Methyloctyl	$C_{26}H_{42}O_4$	antioxidant anti-viral anti-microbial anti-cancer	Aastha Bhardwaj et al 2014
10	Fumaric Acid, 3-Hexyl Tridecyl E...	$C_4H_4O_4$		Anshul Shakya et al 2014				
11	Phthalic Acid, Isobutyl Octadecy	$C_{30}H_{50}O_4$	anti-microbial antioxidant anti-inflammatory	Aastha Bhardwaj et al 2014				
12	Silicic acid diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	anti-cancer	Gershon H, Parmegiani R 1962				

The SVSK2 and SVSK5 strains cell free extracts were used for the identification of anticancer activity against Vero, MCF7 and Hela cell line (general, breast cancer and cervical cancer cell lines). The cytotoxicity assay of bioactive compounds of SVSK2 and SVSK5 showed no harmful effects on normal cell line (Vero), thus indicating these compounds can be used for therapeutic purpose (Fig 4). And the activity was analyzed by dose dependent manner. The IC_{50} value of SVSK2 and SVSK5 cell free extracts for MCF7 and HeLa cells were 150 µg/ml and 300 µg/ml respectively (Fig 4). The cellular morphology of normal cells remain eloquent while the MCF7 and HeLa cells showed reduced growth and disrupted cell wall indicating apoptotic like behavior for both SVSK2 and SVSK5 bioactive compounds. Thus the result of the present study reveals that the bacterial metabolites namely SVSK2 and SVSK5 act as potential compounds for bio-therapeutic treatment.

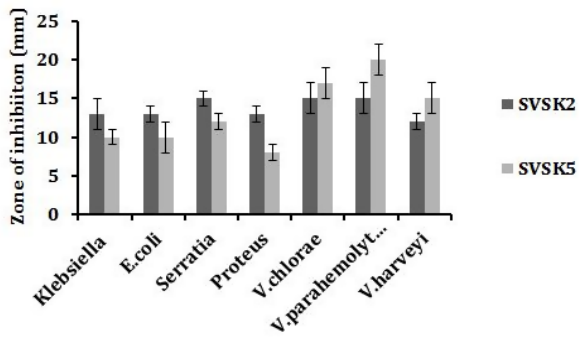


Figure 2: Antimicrobial activity of *B. cereus* SVSK2 and *B. subtilis* SVSK5

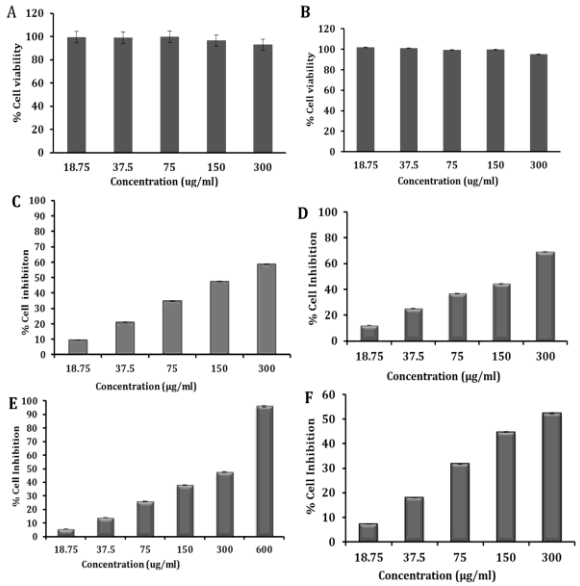
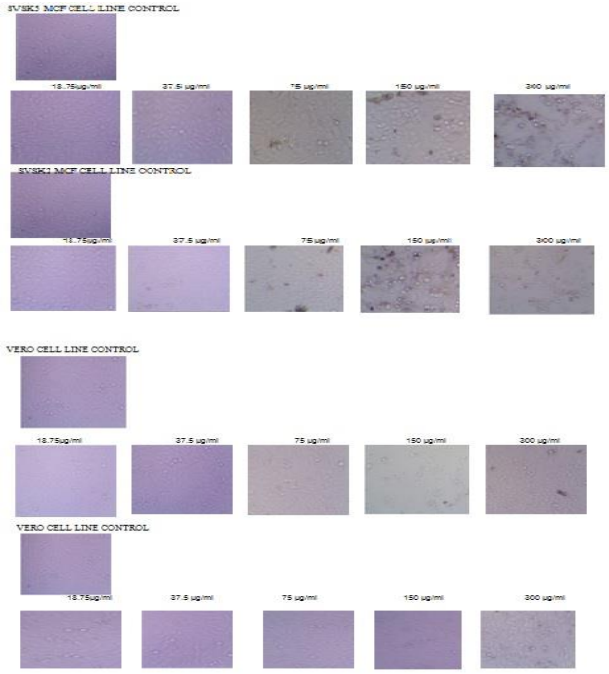


Figure 3: Chemo protective effect of SVSK2 and SVSK5 crude cell free extracts.



Effect of various concentrations of bioactive compounds from *B. cereus* SVSK2 on Vero cell line, (MCF7) Breast cancer cell lines and cervical cancer (HeLa). (A, D & G control, B, E & H -18.75µg, C, F & I-300µg).

Effect of various concentrations of bioactive compounds from *B. subtilis* SVSK5 on Vero cell line, (MCF7) Breast cancer cell lines and cervical cancer (HeLa). (A, D & G control, B, E & H -18.75µg, C, F & I-300µg).

CONCLUSION

The bioactive compounds of isolated probiotic organisms were used as the relative scale to correlate the stress experienced by the fishes through their environmental habitat and food chain. Our study showed promising results to exploit the isolated strains not only as commercial probiotics as supplements and food in aquaculture, but also as a source biochemical substances to synthesize novel therapeutic compounds such as antibiotics and cancer therapeutic agents. For therapeutic purposes, our study lays a rudimentary foundation and further characterization of metabolites and extensive *in vivo* studies may yield interesting results.

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